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(54) Title: SUPPRESSION OF XENOTRANSPLANT REJECTION

(57) Abstract

The invention relates to the suppression of graft rejection, particularly to the suppression of xenograft rejection. In particular, the invention relates to biological tissues that contain endothelial cells that may be induced to generate a compound which down-regulates the expression of a cell adhesion molecule in these cells.

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SUPPRESSION OF XENOTRANSPLANT REJECTION

The present invention relates to the suppression of graft rejection, particularly to the suppression of xenograft rejection.

The success of allogeneic organ transplantation has become well established in the last few decades. However, the limited supply of donor organs means that many patients have little or no chance of receiving a transplanted organ and thus die before a suitable organ is found. One potential solution to this problem is "xenografting", or the use of organs from a non-human ("xenogeneic") animal donor.

Porcine donor organs are particularly suitable candidates for transplantation because pigs are anatomically and physiologically similar to humans, are in abundant supply and are relatively free of pathogens that are capable of causing infections in humans. Furthermore, transgenic technology affords the possibility of genetically modifying the donor tissue to abrogate the rejection response.

One problem associated with xenografting is that xenogeneic organs are rejected rapidly upon re-vascularisation by a humoral process called hyperacute rejection (HAR). This is caused by the presence of naturally-occurring antibodies in the graft recipient, which recognise and react with antigens on the endothelial cells (ECs) of the xenograft. This recognition triggers the complement cascade which in turn leads to rejection.

In the last few years, several novel therapeutic approaches to suppress HAR have been proposed and tested successfully (Bach, 1998). These work either by suppression of complement activation or preventing binding of xenoreactive natural antibodies. HAR is no longer considered an insurmountable problem for pig-to-human transplantation. However, it is becoming clear that preventing HAR alone is unlikely to be sufficient to prevent rejection of xenogeneic organs.

Even if HAR is overcome, vigorous rejection of the graft typically occurs within 2-3 days, much faster than for most forms of allogeneic transplantation, a process termed delayed xenograft rejection (DXR). The histology of this type of rejection is different from HAR with less haemorrhage although with significant intravascular thrombosis. In addition, there is deposition of xenoreactive antibodies on the endothelium along with

fibrin, platelet aggregates and infiltration of the perivascular tissue with inflammatory cells (neutrophils, macrophages and NK cells) (Blakely *et al.*, 1994).

Beyond DXR there is the problem of T-lymphocyte mediated rejection. It has been demonstrated (Dorling *et al.*, 1994) that the T-cell response to porcine xenografts is at least equivalent to the response against allografts, but is likely to be more aggressive and may be difficult to control with standard doses of systemic immunosuppressive drugs.

Endothelial cells (ECs) are thought to orchestrate the recruitment of inflammatory cells in DXR and subsequent cellular rejection in a number of ways: (i) by producing mediators (such as interleukin-8 (IL-8) and platelet activating factor (PAF)) that activate leukocyte function, including adhesion; (ii) by acting as antigen-presenting cells, stimulating the specific immune response against the foreign tissue, and (iii) by regulating the spatial and temporal expression of cell-adhesion molecules to facilitate transmigration of leukocytes into the transplanted organ (Bevilacqua, 1993). Cytokines, released from the recruited leukocytes, dramatically increase the expression of adhesion molecules on the EC surface and enhance the recruitment process.

The first structure that circulating leukocytes come in contact with when blood reperfuses a vascularised graft is the endothelium. The leukocytes must adhere to and cross this endothelial barrier to infiltrate the graft. Recent advances in the understanding of mechanisms of leukocyte-EC interactions have revealed a series of adhesion and activation events (the adhesion cascade) that take place during the emigration of leukocytes into tissues (Springer, 1994). (See Figure 1).

Initial rolling on vascular endothelium is mediated by transient interactions between selectins (e.g. L-selectin on leukocytes, E-selectin on activated EC and P-selectin on both activated EC and activated platelets) and carbohydrate-bearing counter-structures on the opposite cell (EC, leukocyte or platelet) (Tedder *et al.*, 1995). However due to the high on/off rate of these selectin-carbohydrate interactions, this class of receptors cannot support firm adhesion of leukocytes. While rolling, leukocytes become exposed to activating signals which result in an increase in avidity of leukocyte surface integrins. Chemokines are thought to be the likely candidates for this triggering event: IL-8 has been shown to exist anchored to the EC surface via surface proteoglycans (Tanaka *et*

al., 1993) resulting in high local concentrations within the milieu of the rolling leukocyte. This integrin-mediated secondary adhesion results in stable arrest of the leukocyte and is followed by transmigration into the tissues (Springer, 1994).

Members of the immunoglobulin supergene family (IgSF) expressed on the endothelium are counter-receptors for leukocyte integrins. These include vascular cell adhesion molecule (VCAM-1), intercellular adhesion molecules (ICAM-1, ICAM-2), and mucosal vascular addressin (MAdCAM-1). The counter ligands for VCAM-1 are heterodimeric α4 integrins with a non covalent linkage to either a β1 or β7 chain. Integrin α4β1 (very late antigen-4 [VLA-4]) is constitutively expressed on most mononuclear leukocytes including eosinophils, lymphocytes, monocytes, basophils but is absent on neutrophils. In contrast, integrin α4β1 is found primarily on a subset of T cells with a tropism for the intestinal tract and its primary ligand is the mucosal vascular addressin (MAdCAM-1), though it also binds VCAM-1 (18). Leukocyte receptors for ICAM are the β2 integrins 'lymphocyte function associated antigen 1' (LFA-1) and αMβ2 integrin (Mac-1). Neutrophils and all haematopoietic cells (except erythrocytes) express LFA-1, whereas Mac-1 expression is more restricted to monocytes, macrophages and granulocytes.

Quiescent vascular endothelium expresses an array of molecules that should not be sufficient to promote significant binding of leukocytes and subsequent transmigration. However it is well established that peri-transplant organ ischaemia results in endothelial activation with increased expression of adhesion molecules.

The interaction of VCAM-1 on ECs with its ligand, the α4β1 integrin VLA-4, on the leukocyte has recently been shown to be the predominant mechanism triggering arrest of rolling monocytes and lymphocytes, and subsequent spreading (Jones *et al.*, 1994; Alon *et al.*, 1995). Transmigration through endothelial tight junctions involves platelet-endothelial cell adhesion molecule (PECAM, CD31), integrin associated protein (IAP, CD47) and integrin α4β1 (Muller, 1995; Brown, 1996).

It appears that interfering with the recognition processes mediated by these adhesion molecules (in particular ICAM-1, LFA-1, VCAM-1 and CD2) may significantly prolong allogeneic graft survival. Furthermore in some models, not only did adhesion

molecule blockade with monoclonal antibodies induce indefinite graft survival, but also donor-specific tolerance (Isobe *et al.*, 1992).

Porcine ECs have the capacity to mediate both the initial adhesion as well as the migration and activation of infiltrating human leukocytes. Functional interactions between human LFA-1 and pig ICAM, and human VLA-4 and pig VCAM have been documented. Furthermore interaction of human monocytes with porcine endothelium also results in activation of the ECs (Millan *et al.*, 1997). This is likely to promote trafficking of human lymphocytes and monocytes into a porcine xenograft and trigger rejection.

It has been demonstrated that antibodies against porcine adhesion molecules can inhibit the infiltration process (Dorling *et al.*, 1996; Mueller *et al.*, 1995). To inhibit the interaction of VCAM-1 and VLA-4, monoclonal antibodies against either of these molecules have been administered. A VCAM-Ig fusion protein, cyclic peptide antagonists that mimic the α4-integrin binding loop in domain 1 of VCAM-1, and certain naturally-occurring fungal cyclopeptolides have also been used (reviewed in Foster, 1996).

It might be possible to target porcine VCAM-1 specifically with monoclonal antibodies, but it is thought that repeated administration of these antibodies would result in sensitization of the recipient and a decline in efficiency of blockade. Systemic administration of agents such as the VCAM-Ig fusion protein, cyclic peptide antagonists naturally-occurring fungal cyclopeptolides mentioned above would result in blockade not only of the pVCAM-VLA-4 interaction within the graft but also in other tissues of the recipient and may impair the ability of the recipient to fight infections.

There is thus a great need for a method of combating the cellular phase of the rejection process resulting from xenotransplantation, without compromising the immune system of the recipient of the grafted tissue.

SUMMARY OF THE INVENTION

According to the present invention there is provided a biological tissue comprising endothelial cells which may be induced to generate a compound which down-regulates the expression of a cell adhesion molecule by the cells.

The biological tissue may be any tissue suitable for transplantation to a mammal, and includes collections of cells, and individual tissues and organs. Accordingly, this definition includes, fibroblasts, neural tissue, foetal tissue and heart, liver, lung , pancreas, islets, skin, small bowel, cornea, cartilage, bone, muscle, or kidney tissues or organs.

The tissue may be derived from any non-human animal which is sufficiently closely related to the human to allow conservation of function to have been retained. Such animals include sheep, pigs, ratites (ostrich, emu), capybara and primates. The animal of choice is the pig, because their organs have similar physiology and size to human organs. Furthermore, pigs can be bred in large numbers and they are relatively free of pathogens capable of causing infections in humans.

As used herein, the term "expression" may refer to the expression of a peptide or polypeptide from a gene and/or the expression of a peptide or protein on the surface of a cell, as the context requires. By "down-regulates" is meant that the compound acts to decrease the level of expression of the cell adhesion protein. The down-regulation may be at the level of transcription, may be by affecting translation or may act via some other mechanism, for example by effecting changes in mRNA stability.

The cell adhesion molecule to be down-regulated may be any protein that is expressed on the surface of an endothelial cell and which is capable of interaction with a leukocyte or platelet cell, such as VCAM-1, ICAM-1, LFA-1, CD2, PECAM, CD31, IAP, CD47, integrin $\alpha v\beta 3$, MAdCAM, PECAM, selectins (P-, L- and E-selectin), LFA-3, CD80/CD86 or thrombospondin. VCAM-1 is the cell adhesion molecule of choice because interaction of VCAM-1 on ECs with its ligand, the $\alpha 4\beta 1$ integrin VLA-4 on leukocytes has been shown to be the predominant mechanism triggering arrest of rolling monocytes and lymphocytes, and their subsequent spreading.

It is a feature of the present invention that the compound which prevents the expression of the cell adhesion molecule may be inducibly generated. It has been found that targeted disruption of the adhesion molecule VCAM-1 in mice is almost universally fatal for the developing embryo due to a failure of effective placentation, causing absent or delayed chorioallantoic fusion and results in the death of the embryo at between 8 and 12 days *in utero* (Gurtner *et al.*, 1995).

The ideal strategy for induction is that of conditional knockout of the cell adhesion molecule, allowing normal expression during development and in the young adult, but with the ability to inhibit expression of the cell adhesion molecule in the donor organ immediately prior to, and/or following transplantation. Suitable methods of induction will be clear to those of skill in the art, such as cloning the inhibitor compound downstream of a suitable response element, enhancer element or promoter element. For example, several known systems enable the transcription of a gene to be controlled in mammalian cells using small molecules, including the Tet-On™ system (Clontech, UK), the metallothionein promoter system (Palmiter *et al.*, 1983), the ecdysone-inducible mammalian expression system (Invitrogen, BV), steroid-inducible promoters (Clackson *et al.*, 1997) and cytokine inducible promoters (Aranciba *et al.*, 1998; Bachiller *et al.*, 1990). The particular system chosen will be determined by the required degree of suppression.

The compound that down-regulates the expression of the cell adhesion molecule may be a polynucleotide. According to a second aspect of the invention there is provided a biological tissue in which the endothelial cells of the tissue may be induced to generate a polynucleotide which down-regulates the expression of a cell adhesion molecule by the cells.

The polynucleotide may be complementary in sequence to part of the gene or mRNA that encodes the cell adhesion molecule, so that it hybridises to the gene or to the mRNA and so prevents its transcription or translation. Ideally, such a polynucleotide should be of at least 15 nucleotides in length, preferably at least 50 nucleotides, more preferably greater than 100 nucleotides. To ensure that only the cell adhesion gene is targeted, the polynucleotide should be complementary to a portion of the gene that is most divergent from other nucleic acid sequences. The polynucleotide should preferably

hybridise to the gene or to the mRNA encoding the cell adhesion molecule under conditions of high stringency, e.g. 0.1 x SSC, 65°C (where SSC= 0.15M NaCl, 0.015M sodium citrate, pH 7.2).

According to this aspect of the invention, the polynucleotide sequences will act to abrogate transcription of a gene encoding a cell adhesion molecule or translation of a cell adhesion mRNA by hybridising with the molecule, thereby preventing interaction of the nucleic acid with the relevant protein factors necessary for transcription or translation to take place.

Alternatively, the polynucleotide sequences may comprise a ribozyme sequence that specifically targets a gene or mRNA coding for a cell adhesion molecule.

According to a third aspect of the invention there is provided a biological tissue in which the endothelial cells of the tissue may be induced to generate a peptide or polypeptide which down-regulates the expression of a cell adhesion molecule by the cells.

The peptide or polypeptide may possess high affinity for the cell adhesion molecule. Preferably, the affinity of the compound for the cell adhesion molecule is greater that 10^{-8}M, more preferably greater than 10^{-9}M, even more preferably greater than 10^{-10}M. The compound should also exhibit specific binding affinity for the cell adhesion molecule to ensure that binding activity of the compound it is not responsible for inappropriate destruction of other molecules in the cell.

Preferably, the compound is an antibody or antibody fragment, which may be easily prepared with high specificity and affinity for a desired target. Antibody fragments such as Fab fragments or single chain fragments (sFvs) are particularly suitable since they are small molecules with high solubility and greater penetrative capacity in an intracellular environment. Intracellular sFv antibodies have been successfully employed *in vitro* to neutralise viruses (HIV-1 (Rondon *et al.*, 1997) and flaviviruses (Jiang *et al.*, 1995)), and to decrease the expression of intracellular oncoproteins (e.g. erbB-2 (Beerli *et al.*, 1994; Graus Porta *et al.*, 1995) and ras (Bioca *et al.*, 1993)) and cell-surface receptors (e.g. IL-2R (Richardson *et al.*, 1997)). These sFv species were generated from the hybridomas of monoclonal antibodies specific for the target protein.

The polypeptide may be a fusion protein comprising a binding domain that exhibits affinity for a cell adhesion molecule and an effector domain that targets a cell adhesion molecule or that targets a gene or mRNA that codes for the cell adhesion molecule. For example, the effector domain may be delivered (by means of the binding domain) to the environment of the cell adhesion molecule, so that the adhesion molecule is targeted for destruction. If proteinaceous, the effector domain may comprise a protease, a kinase, a phosphatase or any other enzyme that is capable of inactivating a cell adhesion molecule. Alternatively, the effector domain may comprise a oligonucleotide or ribozyme molecule that acts on the gene or mRNA that codes for the cell adhesion molecule.

According to a fourth aspect of the invention there is provided a biological tissue in which the endothelial cells of the tissue may be induced to generate a bispecific fusion protein which down-regulates the expression of one or more cell adhesion molecules by the cells. Such a fusion protein may comprise domains or peptides with affinities for different cell adhesion protein epitopes. For example, a fusion protein could be designed with binding affinity and specificity against two or more different epitopes on a cell adhesion molecule such as VCAM. This would improve efficiency of knockout of the cell adhesion molecule. Furthermore, a number of epitopes could be targeted on different cell adhesion molecules, in order to simultaneously abrogate their expression.

In order that the cell adhesion molecule is not transported to the cell surface for expression, the amino acid sequence of the peptide or polypeptide must include a targeting sequence to direct degradation of the cell adhesion molecule.

The targeting sequence may comprise any suitable intracellular protein trafficking signal, provided that the signal is involved in directing the bound complex to a subcellular compartment for degradation. Examples of suitable intracellular protein trafficking signals are discussed by Pudsley, 1989 and by Magee and Wileman, 1992.

Preferably, the signal comprises a localisation signal that directs a nascent peptide to the endoplasmic reticulum (ER). A particularly suitable signal is the inclusion of the amino acid sequence Lys-Asp-Glu-Leu (KDEL) at the C-terminus of the peptide or polypeptide. Proteins that reside in the lumen of the endoplasmic reticulum (ER), the first compartment for newly made membrane-bound proteins or secreted proteins, are

known to possess this short sequence (Munro and Pelham, 1987). If this sequence is deleted or extended by the addition of further amino acids, the protein is secreted from the cell rather than retained.

Alternative signal regions will be clear to those of skill in the art and include lysosomal targeting sequences. Fusion proteins may also be constructed, comprising a peptide or polypeptide fused to a viral protein such as the HIV-1 *nef* protein, or to cytoplasmic signals for rapid turnover such as are found on CTLA-4 (see Magee and Wileman, (1992) Protein targeting: a practical approach; Oxford University Press).

According to a fifth aspect of the invention there is provided a polypeptide comprising a binding region capable of binding to a cell adhesion molecule and a signalling region for subcellular targeting of the polypeptide. Preferably, the polypeptide comprises an antibody or antibody fragment, most preferably a single chain antibody fragment (sFv). The signalling region of choice is a localisation signal for the endoplasmic reticulum. Most preferably, signalling region comprises the amino acid sequence KDEL at the C terminus of the polypeptide.

According to a sixth aspect of the invention there is provided a polynucleotide encoding a peptide or polypeptide according to the fifth aspect of the invention. Preferably, the polynucleotide will comprise sequences suitable for the regulation of expression of the peptide or polypeptide. This expression can preferably be controlled, such as by cell-specific control, inducible control, or temporal control. Preferably, expression should be specific for vascular smooth muscle cells, fibroblasts, cardiac myocytes, ECs or any combination of these cell types. Most preferably, expression should be specific for ECs; EC specific expression can be achieved by using tissue-specific promoters such as E-selectin, ICAM, and MAdCAM.

According to a seventh aspect of the invention, there is provided a vector comprising a polynucleotide according to the sixth aspect of the invention.

The term "vector" signifies a moiety which is capable of transferring a polynucleotide to a host cell. Preferably the vector is a DNA vector and, more preferably, is capable of expressing RNA encoding a protein according to the invention. Numerous suitable vectors are documented in the art; examples may be found in *Molecular Cloning: a Laboratory*

Manual: 2nd edition, Sambrook *et al.*, 1989, Cold Spring Harbor Laboratory Press or *DNA cloning: a practical approach, Volume II: Expression systems*, edited by D.M. Glover (IRL Press, 1995).

Many known techniques and protocols for the manipulation of nucleic acids, for example, in the preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Short Protocols in Molecular Biology*, Second Edition, Ausubel *et al.* eds., (John Wiley & Sons, 1992) or *Protein Engineering: A practical approach* (edited by A.R. Rees *et al.*, IRL Press 1993). For example, in eukaryotic cells, the vectors of choice may be virus-based.

For certain embodiments of the invention, a bicistronic expression vector can be used in order to allow stoichiometric co-expression of two genes from one mRNA. In one particular system (Jackson *et al.*, 1990), expression is driven from a single promoter and incorporation of the internal ribosome entry site (IRES) of encephalomyocarditis virus (ECMV) allows CAP-independent ribosomal binding and translation of the second open reading frame. This allows transfection of a construct containing sequences directed against two different epitopes on a cell adhesion molecule to improve efficiency of knockout, or allows the targeting of two or more different cell adhesion molecules using only one construct.

Introduction of the nucleic acid may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells under conditions for allowing expression of the gene.

In one embodiment, the nucleic acid of the invention is integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques.

Preferably the vector is suitable for the production of a transgenic animal. Vectors suitable for the generation of transgenic pigs, for example, are described in Heckl-Östreicher (1995), McCurry (1996), White (1995), Yannoutsos (1995), and Langford (1996). Minigene vectors suitable for the generation of transgenic mice are described in Diamond (1995).

According to an eighth aspect of the invention, there is provided a delivery system comprising a molecule of the fifth, sixth or seventh aspects and means to deliver said molecule to a target cell.

The delivery system may be viral or non-viral. Non-viral systems, such as liposomes, avoid some of the difficulties associated with virus-based systems, such as the expense of scaled production, poor persistence of expression, and concerns about safety. Preferably the delivery system is suitable for use in gene therapy. Numerous appropriate delivery systems are known in the art such as, for example, polycationic condensed DNA linked or unlinked to killed adenovirus alone (see Curiel, 1992) and ligand linked DNA (see Wu, 1989). Naked DNA may also be employed, optionally using biodegradable latex beads to increase uptake. Liposomes can also act as gene delivery vehicles encapsulating nucleic acid comprising a gene cloned under the control of a variety of tissue-specific or ubiquitously-active promoters. Mechanical delivery systems such as the approach described in Woffendin *et al* (1994) may also be used.

Direct delivery of gene therapy compositions will generally be accomplished, in either a single dose or multiple dose regime, by injection, either subcutaneously, intraperitoneally, intravenously or intramuscularly or delivered to the interstitial space of a tissue. Other modes of administration include oral and pulmonary administration, using suppositories, and transdermal applications, needles, and gene guns or hyposprays.

Preferably, the delivery system will be targeted so that molecules according to the present invention are taken up by cells suitable for transplantation, or cells which have been transplanted. More preferably the delivery system will be specific for these cells. For example, the delivery system may be targeted to a specific organ, such as the heart or the kidney, or to a specific cell type, such as endothelial cells.

To achieve this the delivery system may, for example, be a receptor-mediated delivery system, being targeted to receptors found on target cells. For example, the delivery system may be targeted to receptors found on heart cells, preferably to receptors found exclusively on heart cells, or it may be targeted to receptors found on endothelial cells, preferably to receptors found exclusively on endothelial cells such as E-selectin or P-selectin.

The delivery system is preferably suitable for the generation of transgenic animals. For example, the delivery system may be targeted to a gamete, a zygote, or an embryonic stem cell.

According to an ninth aspect of the invention, there is provided a method of transfecting a cell with a vector according to the invention. The cell for transfection should be a progenitor of the species which is to be the organ donor, preferably an endothelial cell. The stable transfection of porcine endothelial cells is described in Heckl-Östreicher (1995).

Preferably, the cell is suitable for the generation of a transgenic animal. More preferably, the cell is a gamete, a zygote, or an embryonic stem cell. The transfection of murine ova by microinjection to generate transgenic mice, for example, is described in Diamond (1995), and the microinjection of porcine zygotes, for instance, to generate transgenic pigs is described in Yannoutsos (1995), Langford (1996), and White (1995).

According to a tenth aspect of the invention, there is provided a cell transfected according to the ninth aspect.

According to a eleventh aspect of the invention, there is provided biological tissue comprising a cell according to tenth aspect of the invention. The term "biological tissue" as used herein includes collections of cells, tissues and organs. Accordingly the definition includes, for example, fibroblasts, nervous tissue, heart, liver, or kidney tissues or organs.

According to a twelfth aspect of the invention, there is provided an animal comprising a cell and/or biological tissue according to the invention. Preferably the animal is suitable for the production of organs for transplantation into humans. Preferably the animal is a mammal, and more preferably it is a transgenic pig or a transgenic sheep.

The animal might be treated whilst alive such that it comprises transgenic biological tissue (*ie.* treated by gene therapy). Preferably, a live animal is transfected with a vector which is specifically delivered to endothelial cells to produce transgenic organs suitable for xenotransplantation.

Alternatively, the animal might be born as a transgenic animal. Various suitable approaches for generating such transgenic animals are known in the art (*eg.* Bradley & Liu, 1996; Clarke, 1996; Wheeler, 1994). For example, direct manipulation of the zygote or

early embryo, by microinjection of DNA for instance, is well known, as is the *in vitro* manipulation of pluripotent cells such as embryonic stem cells. Retroviral infection of early embryos has proved successful in a range of species, and adenoviral infection of zona-free eggs has been reported. Transgenesis and cloning of sheep by nuclear transfer has also been described (*eg.* WO97/07668).

According to a thirteenth aspect of the invention, there is provided a method of rendering biological tissue suitable for transplantation, comprising expressing one or more compounds according to the present invention in the biological tissue, preferably exclusively in its endothelial cells. The biological tissue may be so rendered either *in vivo* or *ex vivo*. For example, an animal organ may be *in vivo* transfected with a vector according to the invention, or an organ could be transfected *ex vivo* before transplantation or *in vivo* after transplantation.

According to an fourteenth aspect of the invention, there is provided a method of transplantation comprising transplanting biological tissue according to the invention from a donor animal into a recipient animal. Preferably the method is for xenotransplantation and the donor biological tissue is xenogeneic with respect to the recipient animal.

The invention will now be described in detail with specific reference to single chain antibody fragments directed against the VCAM-1 molecule. It will be appreciated that modification of detail may be made without departing from the scope of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Leukocyte-Endothelial Cell Adhesion cascade

Figure 2: Flow cytometry with phage-antibodies demonstrating specificity for VCAM

Figure 3: Restriction enzyme fingerprinting of the sFv clones

Figure 4: Expression vector for sFv

Figure 5: Cotransfection of sFv/ER with pEF/GFP/ER

Figure 6a: VCAM expression on stable EC transfectants with sFv clone F5

Figure 6b: VCAM expression on stable EC transfectants with sFv clone E6.2

Figure 7a: VCAM expression on stable EC transfectants with sFv clone F5

Figure 7b: VCAM expression on stable EC transfectants with sFv clone E6.2

Figure 8: Staining for VCAM in untransfected and transfected endothelial cells

Figure 9: Binding of Jurkat cells to varying densities of endothelial cells

EXAMPLES

Example 1: Identification of VCAM-specific sFv from a phage-display library

A phage-display antibody library was used to generate an sFv specific for VCAM-1. The library used contains $>10^8$ clones generated using a bank of 50 cloned human VH gene segments with a random nucleotide sequence encoding CDR3 lengths of 4-12 residues (Richardson *et al.*, 1993). This library has already been used to isolate specific single-chain antibodies to a variety of antigens including haptens, foreign and self antigens. However selection has depended upon the availability of purified or recombinant antigen. We developed a novel screening strategy to overcome the lack of recombinant porcine VCAM.

cDNA for porcine VCAM was used to generate stable cell lines that exhibit high levels of surface porcine VCAM expression as assessed by flow cytometry. VCAM positive cells were incubated with 3μM CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate, (Molecular Probes, Oregon) for 1 hour at 37°C. Once this membrane-permeant probe enters the cell esterase hydrolysis converts non-fluorescent CMFDA to fluorescent 5-chloromethylfluorescein which reacts with thiols on proteins to form aldehyde-fixable conjugates. Cells labelled with this are viable and fluorescent for several cell divisions.

A suspension of VCAM-negative cells were incubated with the phage-library in 4% Marvel/PBS at 4°C with gentle agitation. After 30 minutes, the labeled VCAM-positive cells were then added in a 1:10 ratio (VCAM positive:negative) and incubated for a further 90 minutes. The cells were pelleted by centrifugation and washed with PBS three times. The fluorescent VCAM-positive cells (and phage that was bound to the cell surface) were then separated by FACS. Phage were 'rescued' in the standard manner

(Griffiths *et al.*, 1994), amplified and the library was subjected to three further rounds of screening. "Polyclonal" phage ELISA confirmed that the library was enriched for VCAM-specific phage. Individual clones were then tested by flow cytometry for binding to the VCAM-positive cell line and the results from representative clones are shown in Figure 2.

The sequence of the VCAM-specific sFv clones was amplified from minipreps of the phagemid vector by PCR and digested for 18 hours with either *Bst*N1 or *Bsa*J1 according to the manufacturer's protocol. Restriction fragment length polymorphism (RFLP) mapping of the first 15 VCAM-specific clones showed that there were at least 5 distinct patterns of digestion suggesting at least 5 different antibody sequences. (Figure 3). Two of these were used for further analysis.

Example 2: Subcloning of sFv for targeted intracellular expression

Our strategy was to engineer the VCAM-specific sFv to be retained within the ER, so that providing that the sFv-VCAM interaction was of sufficient affinity, both molecules would be retained within the ER and degraded, thereby reducing cell-surface VCAM levels. Initially, the sFv has been expressed using a constitutively active promoter, the promoter from the human elongation factor 1α-subunit (EF-1α).

The sFv was amplified from the phagemid vector by PCR (30 cycles, annealing temperature 55°C, 1.5μM Mg^{2+}) using the primers:

(5')CAGTCTATGCGGCCCCATTCA(3'); and

(5')TCCACAGGCGCGCACTCCCAGCCGGGCATGGCCCAGGT(3').

The resulting fragment was subcloned into *Bss*HII/*Not*I sites in pEF/*myc*/ER (Invitrogen, BV). The sFv was directed to the ER by incorporation of the sequence of a signal peptide from a mouse VH chain at the 5'-end of the sFv gene; this peptide is cleaved upon translocation into the ER. The sFv is retained because of the KDEL peptide sequence at the C-terminus. The construct is shown diagramatically in Figure 4.

Example 3: Effect of sFv constructs on VCAM expression

Co-transfection of sFv/ER with pEF/GFP/ER

Functional analysis of the constructs was carried out by transfection into an immortalized porcine endothelial cell line A9. These were generated by microinjection of pZipSVU19 DNA into primary aortic endothelial cells (Dorling *et al.*, 1996). The immortalized cells retain the characteristics of endothelial cells but unlike primary ECs, demonstrated constitutive expression of VCAM. Cytokine treatment of the cells increased VCAM expression marginally (RMFI increase from 38.2 to 66.3 at 72 hours).

Transfection of DNA was carried out with the liposome formulation LipofectAMINE (Life Technologies) using a modification of the manufacturer's protocol. LipofectAMINE reagent is a 3:1 (w/w) formulation of the polycationic lipid 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidtlethanolamine (DOPE) in water.

1 x 10^5 cells were seeded in each well of a 6-well plate in complete medium (DMEM) and allowed to attach overnight at 37°C, in a 5% CO_2 incubator overnight. The following day the cells were washed twice in pre-warmed serum-free Opti-MEM® I (Gibco BRL). 2 mL of serum-free Opti-MEM® I was added to each well and the cells returned to the 37°C CO_2 incubator for 2-3 hours.

DNA to be transfected (1-2µg per transfection) was diluted to a final volume of 100µL per transfection in serum-free medium, Opti-MEM® I. To a separate tube, for each transfection, 5µL LiopfectAMINE and 15µg Bovine Transferrin (Sigma; stock solution made up as 1µg/µL in serum-free Opti-MEM® I) were added to a final volume of 100µL in serum-free Opti-MEM® I. The DNA and the LipofectAMINE-transferrin mix were combined and left to stand at room temperature for 30 minutes to allow DNA-liposome complex formation.

For each transfection, 0.8mL of prewarmed serum-free Opti-MEM® I was added to the tube containing the complexes. The medium was aspirated from the walls and 1mL of the diluted complex solution was added to the cells. The cells were incubated at 37°C in 5% CO_2 for 5-6 hours. The transfection mixture was then removed and 2mL of complete medium (DMEM) containing 10% FCS added to each well.

In transient transfection assays, the cells were assayed 24-72 hours after the start of transfection. The transfected cells were harvested and stained with the monoclonal antibody 10.2C7 specific for porcine VCAM (Celltech, UK) and a second layer reagent labeled with Texas red. In order to identify cells which had taken up the plasmid DNA, the sFv constructs were co-transfected with the vector pEF/GFP/ER which contains a 716bp fragment from pαGFP (Crameri *et al.*, 1996) fused to the KDEL ER-retention signal in the same vector backbone as the sFv constructs.

Figure 5 shows a typical histogram when the population was assessed for VCAM expression (red fluorescence) and GFP expression (green fluorescence). Cells expressing GFP showed a 77% reduction in VCAM expression (RMFI control 10.9, GFP positive cells 2.4). This reduction was not seen in cells transfected with the pEF/GFP/ER plasmid alone suggesting the reduction was due to the expression of the VCAM-specific sFv.

Example 4: Analysis of stable transfectants

To obtain stable transfectants, IPEC were co-transfected with a plasmid encoding hygromycin resistance (Kioussis *et al.*, 1987). After 48-72 hours the cells were passaged and diluted 1:10 into complete medium containing 150μg/mL hygromycin. VCAM expression was analyzed by flow cytometry of the cells resistant to hygromycin at 10-14 days.

Figures 6 A and B show typical histograms of cell lines generated by transfection of constructs of two different sFv clones, E6.2 and F5. Both sFv constructs reduce VCAM expression on the surface of the endothelial cells. The values for mean fluorescence corresponding to the level of VCAM expression demonstrated in these Figures are: RMFI untransfected population = 38.2, clone sFv/ER E6.2 = 4.82 and F5 = 10.1.

Figures 7 A and B present the results of a second experiment in permanently transfected cells to assess the reduction of VCAM expression on the surface of these cells.

These data demonstrate that it is possible to reduce VCAM expression on endothelial cells by expressing within the cells a VCAM-specific scFv targeted for retention within the ER.

Example 5: Transfection of scFv-ER constructs traps VCAM within the cell.

The techniques of immunofluorescence staining and confocal microscopy were then used to demonstrate co-localisation of VCAM and scFv within both the E6.2 and F5 scFv transfectants.

Untransfected A9 endothelial cells demonstrate diffuse membrane staining for VCAM (see Figure 8A). Both scFv transfected clones demonstrated a perinuclear, punctuate staining pattern when stained for VCAM expression, consistent with retention of VCAM within the endoplasmic reticulum (an example of one transfected cell is shown in Figure 8B). Staining the transfected cells with an anti-myc antibody to detect the c-myc epitope on the scFv constructs produced a strong perinuclear staining pattern consistent with ER-retention of the scFv (Figure 8C). Dual exposure with appropriate filters confirmed that the VCAM and scFv were co-localised in the ER (Panel D).

Example 6: Reduction of VCAM expression by intracellular scFv is associated with reduction in adherence of human leukocytes

In order to demonstrate that reduction in VCAM expression achieved by the intracellular scFv was functionally significant, binding of the T cell leukaemia line, Jurkat, was examined, both to the transfectants and to the parent A9 endothelial cell line.

The Jurkat line J6 was chosen because of high expression of surface VLA-4 and documented reliance on this integrin for binding to endothelial cells (van Kooyk, Y *et al*; 1993).

The graphs shown in Figures 9A and B demonstrate the binding of Jurkat cells to varying densities of endothelial cells plated in individual wells of a 96 well plate. For both scFv transfectants, there was a right shift in the binding curve demonstrating a reduced affinity of the Jurkat for the transfectants, as well as a significant reduction in the maximal binding of the leukocytes to the endothelial cell monolayer.

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CLAIMS

1. A biological tissue comprising endothelial cells which may be induced to generate a compound which down-regulates the expression of a cell adhesion molecule by the cells.

2. A tissue according to claim 1 wherein said compound which down-regulates the expression of a cell adhesion molecule by the cells is a polynucleotide.

3. A tissue according to claim 1 or 2 wherein said compound which down-regulates the expression of a cell adhesion molecule by the cells is a peptide or polypeptide.

4. A tissue according to claim 3 wherein said polypeptide is a bispecific fusion protein.

5. A polypeptide comprising a binding region capable of binding to a cell adhesion molecule and a signalling region for subcellular targeting of the polypeptide.

6. A polypeptide according to claim 5 which comprises an antibody.

7. A polypeptide according to claim 6 which comprises a fragment of an antibody.

8. A polypeptide according to claim 7 which comprises a single chain Fv fragment.

9. A polypeptide according to any of claims 5 to 8, wherein the signalling region for subcellular targeting of the polypeptide comprises a localisation signal for the endoplasmic reticulum.

10. A polypeptide according to claim 9, wherein the signalling region comprises the amino acid sequence KDEL at the C terminus of the polypeptide.

11. A polypeptide according to any one of claims 5 to 10, wherein said binding region has affinity for any one of the adhesion molecules VCAM-1, ICAM-1, LFA-1, CD2, PECAM, CD31, IAP, CD47 or integrin $\alpha v\beta 3$.

12. A polynucleotide encoding a peptide or polypeptide according to any one of claims 5 to 11.

13. A vector comprising a polynucleotide according to claim 12.

14. A cell comprising a polynucleotide according to claim 12.

15. Biological tissue comprising a cell according to claim 14.

16. An animal comprising biological tissue according to claim 15 and/or a cell according to claim 14.

17. An animal according to claim 16, wherein said animal is a transgenic pig or sheep.

18. A method of rendering a tissue or organ suitable for transplantation, comprising expressing a polypeptide according to any one of claims 5 to 11 in endothelial cells in said tissue or organ.

19. A method of transplantation comprising transplanting biological tissue according to claim 15 from a donor animal into a recipient animal.

FIG. 1

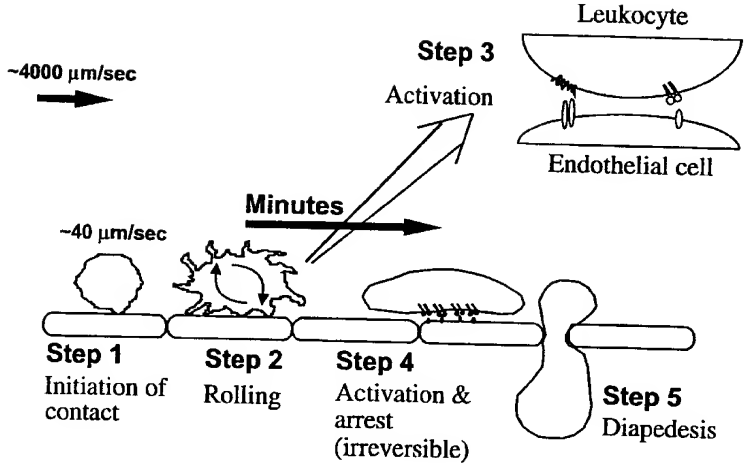
Leukocyte
Step 3
Activation
~4000 µm/sec
Endothelial cell
Minutes
~40 µm/sec
Step 1
Initiation of contact
Step 2
Rolling
Step 4
Activation & arrest (irreversible)
Step 5
Diapedesis

FIG. 2

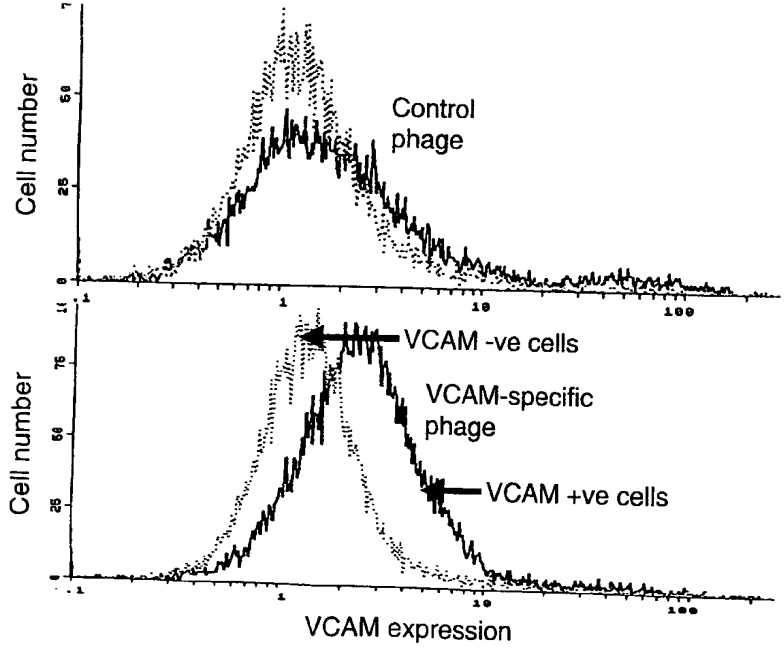
Cell number
Control phage
VCAM -ve cells
VCAM-specific phage
VCAM +ve cells
Cell number
VCAM expression

FIG. 3

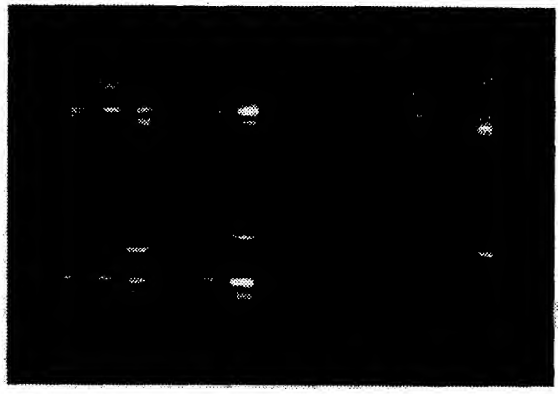

FIG. 4

ER retention with SEKDEL signal

For stable clones, Hyg-resistance plasmid cotransfected

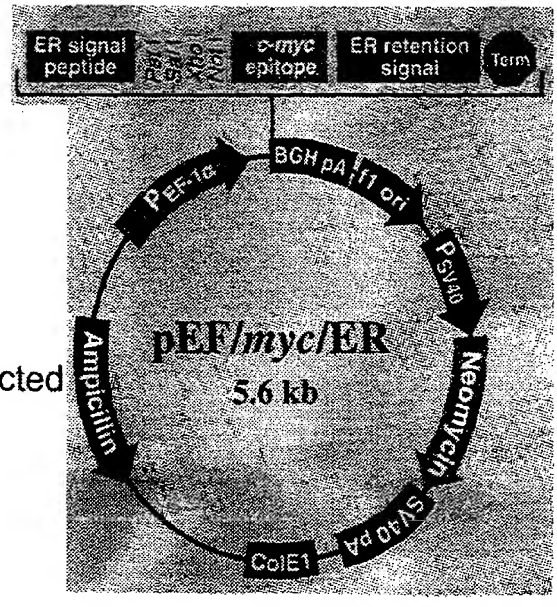

FIG. 5

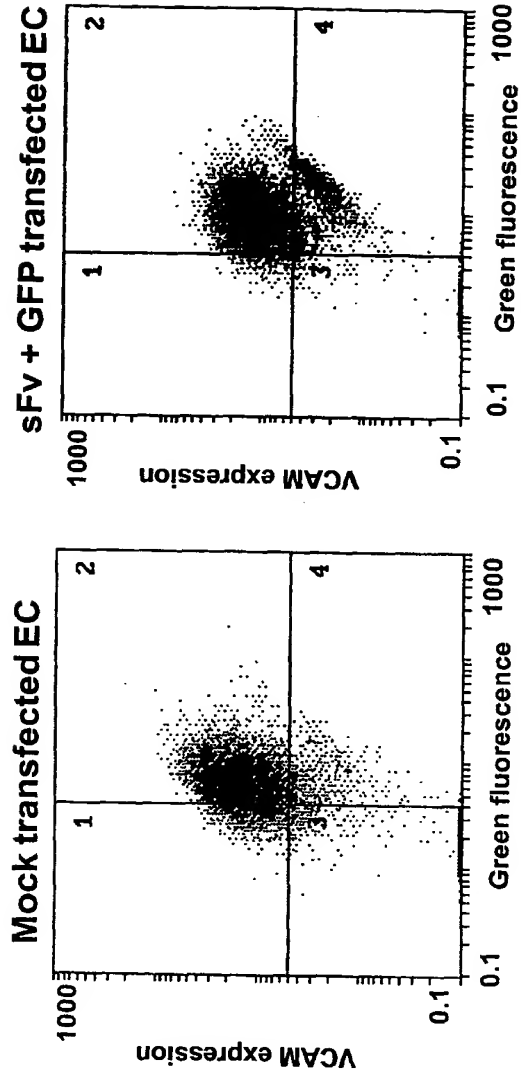
Mock transfected EC
sFv + GFP transfected EC
VCAM expression
Green fluorescence
1000
0.1
1
2
3
4

FIG. 6a

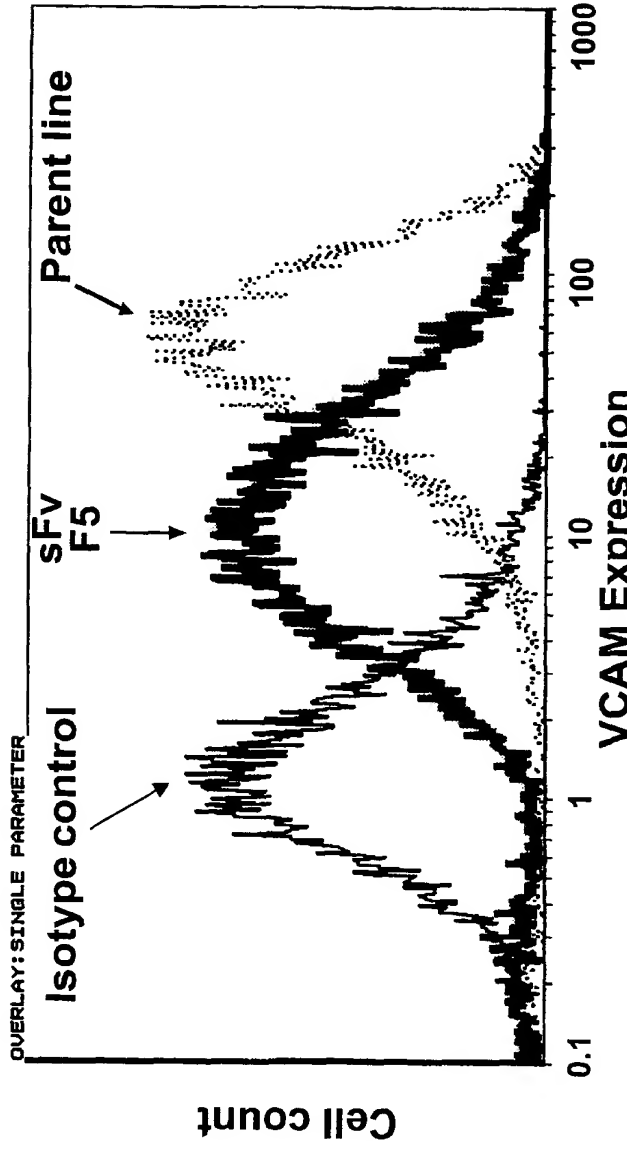
OVERLAY: SINGLE PARAMETER
Isotype control
sFv F5
Parent line
Cell count
0.1
1
10
100
1000
VCAM Expression

FIG. 6b

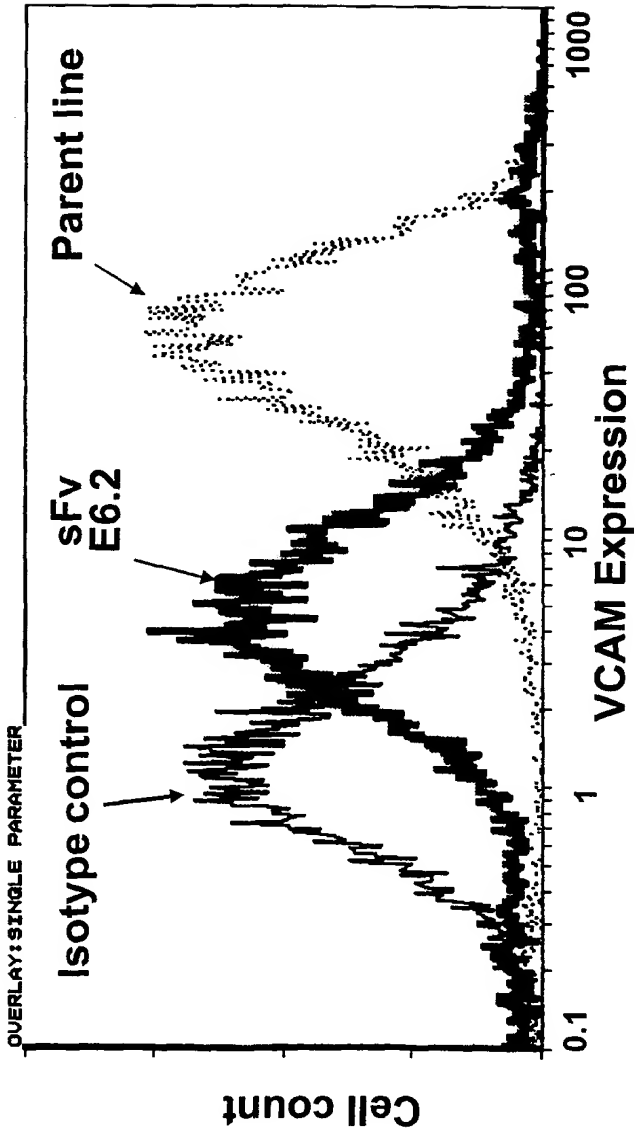
OVERLAY: SINGLE PARAMETER
Isotype control
sFv E6.2
Parent line
Cell count
0.1
1
10
100
1000
VCAM Expression

FIG. 7A

Clone E6a

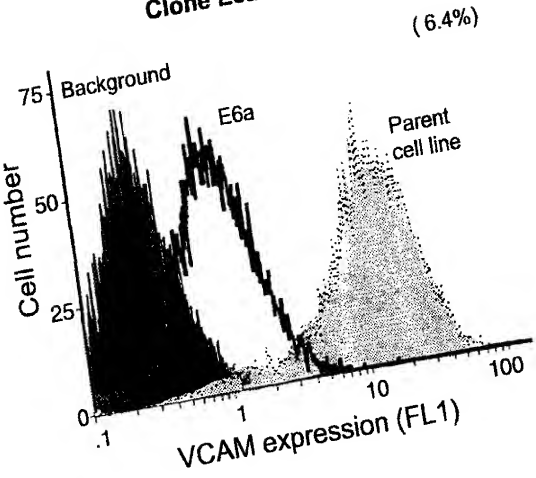

FIG. 7B

Clone F5a

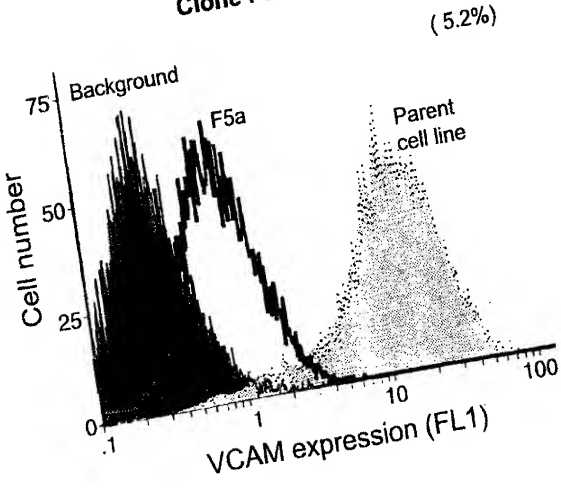

FIG. 8A

FIG. 8B

FIG. 8C

FIG. 8D

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
P020378WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 99/03888	22/11/1999	20/11/1998

Applicant

IMPERIAL COLLEGE INNOVATIONS LIMITED et al.

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [X] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

ational application No.
PCT/GB 99/03888

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 18 and 19 are directed to a method of treatment or a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTER ONAL SEARCH REPORT

Information on patent family members

Int[redacted]onal Application No
PCT/GB 99/03888

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9615780 A	30-05-1996	US 5883082 A	16-03-1999
		AU 4371296 A	17-06-1996
WO 9711971 A	03-04-1997	AU 7378096 A	17-04-1997
		CA 2232937 A	03-04-1997
		EP 0866807 A	30-09-1998

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	ISOBE M ET AL: "SPECIFIC ACCEPTANCE OF CARDIAC ALLOGRAFT AFTER TREATMENT WITH ANTIBODIES TO ICAM-1 AND LFA1" SCIENCE,US,AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, vol. 255, 28 February 1992 (1992-02-28), pages 1125-1127, XP002056591 ISSN: 0036-8075 cited in the application the whole document	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	GERRITSEN M E ET AL: "FLAVENOIDS INHIBIT CYTOKINE-INDUCED ENDOTHELIAL CELL ADHESION PROTEIN GENE EXPRESSION" AMERICAN JOURNAL OF PATHOLOGY,US,PHILADELPHIA, PA, vol. 147, no. 2, 1 August 1995 (1995-08-01), pages 278-292, XP000575649 ISSN: 0002-9440 the whole document	1
X	ELICES M J ET AL: "VCAM-1 ON ACTIVATED ENDOTHELIUM INTERACTS WITH THE LEUKOCYTE INTEGRIN VLA-4 AT A SITE DISTINCT FROM THE VLA-4/FIBRONECTIN BINDING SITE" CELL,US,CELL PRESS, CAMBRIDGE, NA, vol. 60, 23 February 1990 (1990-02-23), pages 577-584, XP000606922 ISSN: 0092-8674 the whole document	5,11-17
P,X P,A	ROBINSON L A ET AL: "The role of adhesion molecules in human leukocyte attachment to porcine vascular endothelium: implications for xenotransplantation." JOURNAL OF IMMUNOLOGY, (1998 DEC 15) 161 (12) 6931-8. , XP000887398 the whole document	5,11-17
A	WO 96 15780 A (ISIS PHARMACEUTICALS INC ;UNIV TEXAS (US); BENNETT C FRANK (US); S) 30 May 1996 (1996-05-30) the whole document	
A	WO 97 11971 A (ALEXION PHARMA INC) 3 April 1997 (1997-04-03) page 14, line 20 -page 16, line 30	
A	HAMMER C ET AL: "Organs from animals for man." INTERNATIONAL ARCHIVES OF ALLERGY AND IMMUNOLOGY MAY, 1998, vol. 116, no. 1, May 1998 (1998-05), pages 5-21, XP000909785 ISSN: 1018-2438 page 11, column 1, paragraph 6 -column 2, paragraph 3	

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INTER ONAL SEARCH REPORT

Int[redacted]onal Application No
PCT/GB 99/03888

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/10 C07K16/46 C12N15/62 A01K67/027 C12N15/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DORLING ANTHONY ET AL: "In vitro accommodation of immortalized porcine endothelial cells: Resistance to complement mediated lysis and down-regulation of VCAM expression induced by low concentrations of polyclonal human IgG antipig antibodies." TRANSPLANTATION (BALTIMORE) 1996, vol. 62, no. 8, 1996, pages 1127-1136, XP000909707 ISSN: 0041-1337 the whole document --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 May 2000	19. 06. 00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Bilang, J

Form PCT/ISA/210 (second sheet) (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P020378WO PNH		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/03888	22/11/1999	20/11/1998
International Patent Classification (IPC) or national classification and IPC C07K14/00		
Applicant IMPERIAL COLLEGE INNOVATIONS LIMITED et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
20/06/2000	08.03.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Bilang, J Telephone No. +49 89 2399 8707

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/03888

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-23 as originally filed

Claims, No.:

1-16 with telefax of 23/02/2001

Drawings, sheets:

1/9-9/9 as originally filed

Sequence listing part of the description, pages:

1-2, filed with the letter of 26.1.00

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☒ furnished subsequently to this Authority in written form.
   - ☒ furnished subsequently to this Authority in computer readable form.
   - ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-16
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-16
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-16
	No:	Claims	

2. Citations and explanations
   see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Additional remarks Item I

The priority appears to be validly claimed.

Additional remarks Item V

1. The present application discloses endothelial cells capable of expressing a compound which down-regulates the expression of cell adhesion molecules (CAM). Said compound may be an antisense oligonucleotide or a fusion peptide capable of binding the CAM. The expression of the compound is under the control of an inducible promoter.

2. The present International Preliminary Examination Report is based on the following documents:

   D1: Dorling et al., Transplantation, vol. 62, no. 8, 1996, p. 1127-1136
   D2: Gerritsen et al., American J. of Pathology, vol. 147, no. 2, 1995, p. 278-292
   D3: Elices et al., Cell, vol. 60, issue of 23.02.1990, p. 577-584
   D4: WO96/15780
   D5: WO97/11971
   D6: Isobe et al., Science, vol.255, issue of 28.02.1992, p. 1125-1127

3. It was generally recognized that cell adhesion is a mayor factor in xenograft rejection (D4-D6). The corresponding molecules were also known. Prior art suggests treatment with antibodies against the cell adhesion molecules (D6), antisense approaches (administration of the antisense molecules to the patient; D4, D5) or knock-out of the cell adhesion genes (D5). However, none of the available prior art documents appears to refer to inducible expression of the antisense or of a protein capable of binding to the cell adhesion molecules in the (donor) tissue.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 27 July 2000 (27.07.00)	
International application No. PCT/GB99/03888	**Applicant's or agent's file reference** P020378WO
International filing date (day/month/year) 22 November 1999 (22.11.99)	**Priority date** (day/month/year) 20 November 1998 (20.11.98)
Applicant RAMRAKHA, Punit, Satyavrat et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

20 June 2000 (20.06.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Pascal Piriou

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

HOWARD, Paul, Nicholas
Carpmaels & Ransford
43 Bloomsbury Square
London WC1A 2RA
ROYAUME-UNI

Date of mailing (day/month/year)	
21 August 2000 (21.08.00)	
Applicant's or agent's file reference	**IMPORTANT NOTIFICATION**
P020378WO	
International application No.	**International filing date (day/month/year)**
PCT/GB99/03888	22 November 1999 (22.11.99)

1. The following indications appeared on record concerning:

[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [X] the name [X] the address [X] the nationality [X] the residence

Name and Address	State of Nationality	State of Residence
DORLING, Anthony Imperial College School of Medicine Hammersmith Hospital Du Cane Road London W12 0NN United Kingdom	GB	GB
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

3. Further observations, if necessary:
Additional applicant/inventor for the purposes of US only.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Dominique DELMAS
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

CLAIMS

1. A biological tissue comprising endothelial cells which may be induced to generate a compound which down-regulates the expression of a cell adhesion molecule by the cells.

2. A tissue according to claim 1 wherein said compound which down-regulates the expression of a cell adhesion molecule by the cells is a polynucleotide.

3. A tissue according to claim 1 or 2 wherein said compound which down-regulates the expression of a cell adhesion molecule by the cells is a peptide or polypeptide.

4. A tissue according to claim 3 wherein said polypeptide is a bispecific fusion protein.

5. A polypeptide comprising a binding region capable of binding to a cell adhesion molecule and a signalling region for subcellular targeting of the polypeptide.

6. A polypeptide according to claim 5 which comprises an antibody.

7. A polypeptide according to claim 6 which comprises a fragment of an antibody.

8. A polypeptide according to claim 7 which comprises a single chain Fv fragment.

9. A polypeptide according to any of claims 5 to 8, wherein the signalling region for subcellular targeting of the polypeptide comprises a localisation signal for the endoplasmic reticulum.

10. A polypeptide according to claim 9, wherein the signalling region comprises the amino acid sequence KDEL at the C terminus of the polypeptide.

11. A polypeptide according to any one of claims 5 to 10, wherein said binding region has affinity for any one of the adhesion molecules VCAM-1, ICAM-1, LFA-1, CD2, PECAM, CD31, IAP, CD47 or integrin $\alpha v\beta 3$.

12. A polynucleotide encoding a peptide or polypeptide according to any one of claims 5 to 11.

13. A vector comprising a polynucleotide according to claim 12.

14. A cell comprising a polynucleotide according to claim 12.

15. Biological tissue comprising a cell according to claim 14.

16. An animal comprising biological tissue according to claim 15 and/or a cell according to claim 14.

17. An animal according to claim 16, wherein said animal is a transgenic pig or sheep.

18. A method of rendering a tissue or organ suitable for transplantation, comprising expressing a polypeptide according to any one of claims 5 to 11 in endothelial cells in said tissue or organ.

19. A method of transplantation comprising transplanting biological tissue according to claim 15 from a donor animal into a recipient animal.